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(54) Title: CHIMERIC FATTY BODY-PRO-GRF ANALOGS WITH INCREASED BIOLOGICAL POTENCY		
(57) Abstract <p>The present invention relates to chimeric fatty body-pro-GRF analogs with increased biological potency, their application as anabolic agents and in the diagnosis and treatment of growth hormone deficiencies. The chimeric fatty body-pro-GRF analogs include a hydrophobic moiety (tail), and can be prepared, either by anchoring one or several hydrophobic tails to the GRF, or by substituting one or several amino-acids by a pseudomicellar residue in the chemical synthesis of GRF. The GRF analogs of the present invention are biodegradable, non-immunogenic and exhibit an improved anabolic potency with a reduced dosage and prolonged activity.</p>		

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**CHIMERIC FATTY BODY-PRO-GRF ANALOGS WITH INCREASED
BIOLOGICAL POTENCY**

BACKGROUND OF THE INVENTION

5 (a) Field of the Invention

The invention relates to chimeric fatty body-pro-GRF analogs with increased biological potency and prolonged activity, their application as anabolic agents and treatment of growth hormone deficiencies.

10 (b) Description of Prior Art

Growth hormone (GH) or somatotropin, secreted by the pituitary gland constitute a family of hormones which biological activity is fundamental for the linear growth of a young organism but also for the maintenance of the integrity at its adult state. GH acts directly or indirectly on the peripheral organs by stimulating the synthesis of growth factors (insulin-like growth factor-I or IGF-I) or of their receptors (epidermal growth factor or EGF). The direct action of GH is of the type referred to as anti-insulinic, which favors the lipolysis at the level of adipose tissues. Through its action on IGF-I (somatomedin C) synthesis and secretion, GH stimulate the growth of the cartilage and the bones (structural growth), the protein synthesis and the cellular proliferation in multiple peripheral organs, including muscles and the skin. Through its biological activity, GH participates within adults at the maintenance of a protein anabolism state, and plays a primary role in the tissue regeneration phenomenon after a trauma.

The decrease of GH secretion with the age, demonstrated in humans and animals, favors a metabolic shift towards catabolism which initiates or participate to the aging of an organism. The loss in muscle mass, the accumulation of adipose tissues, the bone demineralization, the loss of tissue regeneration capacity

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after an injury, which are observed in elderly, correlate with the decrease in the secretion of GH.

GH is thus a physiological anabolic agent absolutely necessary for the linear growth of children and
5 which controls the protein metabolism in adults.

The secretion of GH by the pituitary gland is principally controlled by two hypothalamic peptides, somatostatin and growth hormone-releasing factor (GRF). Somatostatin inhibits its secretion, whereas GRF stimulates it.
10

The human GH has been produced by genetic engineering for about ten years. Until recently most of the uses of GH were concerned with growth delay in children and now the uses of GH in adults are studied.
15 The pharmacological uses of GH and GRF may be classified in the following three major categories.

Children growth

Treatments with recombinant human growth hormone have been shown to stimulate growth in children
20 with pituitary dwarfism, renal insufficiencies, Turner's syndrome and short stature. Recombinant human GH is presently commercialized as an "orphan drug" in Europe and in the United States for children's growth
25 retardation caused by a GH deficiency and for children's renal insufficiencies. The other uses are under clinical trial investigation.

Long term treatment for adults and elderly patients

30 A decrease in GH secretion causes changes in body composition during aging. Preliminary studies of one-year treatment with recombinant human GH reported an increase in the muscle mass and in the thickness of skin, a decrease in fat mass with a slight increase in
35 bone density in a population of aged patients. With respect to osteoporosis, recent studies suggest that

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recombinant human GH does not increase bone mineralization but it is suggested that it may prevent bone demineralization in post-menopausal women. Further studies are currently underway to demonstrate this theory.

Short term treatment in adults and elderly patients

In preclinical and clinical studies, growth hormone has been shown to stimulate protein anabolism and healing in cases of burn, AIDS and cancer, in wound and bone healing.

GH and GRF are also intended for veterinary pharmacological uses. Both GH and GRF stimulate growth in pigs during its fattening period by favoring the deposition of muscle tissues instead of adipose tissues and increase milk production in cows, and this without any undesired side effects which would endanger the health of the animals and without any residue in the meat or milk being produced. The bovine somatotropin (BST) is presently commercialized in the United States.

Most of the clinical studies presently undertaken were conducted with recombinant GH. The GRF is considered as a second generation product destined to replace in the near future the uses of GH in most instances. Accordingly, the use of GRF presents a number of advantages over the use of GH per se.

Physiological advantages

Growth hormone (GH) is secreted by the pituitary gland in a pulse fashion, since this rhythm of secretion is crucial for an optimal biological activity. The administration of GH to correspond to its natural mode of secretion is difficult to achieve. When GRF is administered in a continuous fashion as a slow releasing preparation or as an infusion, it

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increases GH secretion while respecting its pulsatility.

The recombinant GH which is presently commercialized is the 22 kDa form whereas GRF induces the synthesis and secretion from the pituitary gland of all the chemical isomers of GH which participate in a wider range of biological activities.

A treatment with GH results in a decreased capacity of the pituitary gland to secrete endogenous growth hormone, and the GH response to GRF is diminished after such a treatment. On the contrary, a treatment with GRF does not present this disadvantages, its trophic action on the pituitary gland increases this gland secreting capacity in normal animals and in patients with somatotroph insufficiency.

Economical advantages

The production of GH by genetic engineering is very expensive for clinical use. In particular, there are risks of contamination of these commercial preparation with material from the bacterial strain used. These bacterial contaminants may be pyrogens or may result in immunogenic reactions in patients. The purification of the recombinant product is effected by following a plurality of successive chromatography steps. The drastic purity criteria causes multiple quality control steps.

The synthesis of GRF is of chemical nature. The synthesis effected in a solid phase and its purification is carried out in a single step using high performance liquid chromatography (HPLC). Also the quantity of GRF to be administered is much less than the quantity of GH for the same resulting biological activity.

Even with all these advantages, GRF is still not commercialized to date as a therapeutic agent

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mainly because of its chemical instability. The human GRF is a peptide of 44 amino acids of the following sequence:

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5  Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
    1      5      10      15
    Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg Gln Gln Gly
      20      25      30
10  Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu-NH2
    35      40      (SEQ ID NO:1).

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The minimum active core is hGRF (1-29)NH₂

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    Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
    1      5      10      15
15  Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg
      20      25      (SEQ ID NO:2).

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As for many peptides, hGRF (1-29)NH₂ is rapidly degraded in a serum medium and its metabolites have no residual biological activity. It has been well established that the action of enzymes, namely that of dipeptidylaminopeptidase type IV, in a blood medium results in the hydrolysis of the peptide bond Ala²-Asp³ of GRF. This hydrolysis results in a multitude of negative consequences which was the subject of many studies reported in the literature. Essentially, this hydrolysis leads to the formation of truncated peptides of specific activity reduced to less than 1/1000 of the biological activity.

Clinical studies with children and adults have confirmed that natural hGRF (1-44)NH₂ or the active fragment hGRF (1-29)NH₂ are not potent enough to produce equal effects corresponding to those of recombinant GH.

Many GRF analogs have been described, but they all present the disadvantages of being modified GRF having a different amino acid sequence or having synthetic amino acids (D series) added. These GRF analogs

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are potentially immunogenic and their administration to human may cause immunotoxicity problems and potential side effects.

It is well known that the anchoring of hydrophobic groups, such as -NEt₂ at the C-terminal of a peptidic sequence can result in a significantly increased specific activity. In terms of hydrophobicity, these results are contradicted by a fare number recent works such as those of Muranichi (S. Muranichi et al., 1991, *Pharm. Res.*, 8:649-652) which stress the inefficacy of the lauroyl group as an hydrophobic group used in the synthesis of small peptides analogs. Hence, the contradictory investigations of the prior art failed to address the issue of finding a more potent GRF analog using hydrophobic residues.

Gaudreau et al. (P. Gaudreau et al., 1992, *J. Med. Chem.*, 35(10),:1864-1869) describe the affinity of acetyl-, 6-aminohexanoyl-, and 8-aminooctanoyl-GRF(1-29)NH₂ with the rat pituitary receptor. In this report, none of the fatty acid-GRF compounds tested exhibited a higher affinity than hGRF(1-29)NH₂ itself, and the authors concluded that "...modifications to increase the hydrophobic character at the N-terminus of hGRF(1-29)NH₂ do not constitute a suitable approach to increase receptor affinity."

Coy et al. (D.H. Cow et al., 1987, *J. Med. Chem.*, 30:219-222) describe an acetyl-GRF peptide with an increased biological activity on a rat model, more particularly on a rat anesthetized with sodium pentobarbital. The in vitro GH response by cultured rat pituitary cells was also analyzed. However, these authors did not synthesize and test fatty acid-GRF analogs with a carbon chain longer than 2 (acetyl) added at the N-terminus region of the GRF.

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Up to now, most of the GRF analogs described (including those of Gaudreau et al. and those of Coy et al.) have been tested in rat models, either *in vitro* or *in vivo*. Since human and rat GRF(1-29)NH₂ are markedly different, the structure-activity relationships of GRF is different in both species. Therefore, it is not possible to extrapolate results obtained in rats to human.

Accordingly, it is necessary to design GRF analogs with improved anabolic potency and having a prolonged activity. This increased potency could result from a resistance to serum degradation and/or from hyperagonistic properties.

It would be highly desirable to be provided with GRF analogs with increased anabolic potency, while remaining biodegradable and structurally closed to natural GRF, in order to prevent immune reactions when chronically injected in humans and animals.

20 SUMMARY OF THE INVENTION

One aim of the present invention is to provide new biodegradable and non-immunogenic pro-GRF analogs with improved biological potency and prolonged activity.

25 Another aim of the present invention is to provide pro-GRF analogs with increased anabolic potency and prolonged activity, i.e. capable to substantially elevate insulin-like growth factor I (IGF-I) levels when chronically administered in human and animals.

30 Another aim of the present invention is to provide a mean to render any pro-GRF analog more biologically potent and with a prolonged activity.

Another aim of the present invention is to provide for a method of producing active pro-GRF analogs with improved anabolic potency and prolonged activity.

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The present invention relates to the preparation of chimeric fatty body-GRF analogs. These chimeric analogs include an hydrophobic moiety (tail), and can be prepared, either by anchoring one or several hydrophobic tails to the GRF, or by substituting one or several amino-acids by a pseudomicellar residue in the chemical synthesis of pro-GRF. The pro-GRF analogs in accordance with the present invention are characterized in that:

- 10 a) These analogs possess an enhanced biological activity; specifically, they are able to markedly increase GH and IGF-I blood levels when administered in an animal model closely related to human. This characteristic is particularly advantageous in that it results in a reduced dosage of an hyperactive compound being administered to the patient, thus improving treatment efficacy and reducing treatment costs.
- 15 b) Both natural amino acid and hydrophobic metabolisable substances, such as fatty acids, are used for the chemical synthesis of the pro-GRF analogs. Such a use of natural substances entirely metabolisable is intended to prevent the potential secondary effects, namely in cases of multiple administrations.
- 20 c) They present a high biological activity at infinitely small dosages.
- 25 d) They remain active for a prolonged period of time, with a high biological activity.

30 The use of fatty bodies in accordance with the present invention results in pro-GRF analogs which overcome all the drawbacks of the prior art. The pro-GRF analogs of the present invention are biodegradable, non-immunogenic and exhibit an improved anabolic potency with a reduced dosage and have a prolonged

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activity. Furthermore, the present invention deals with GRF and any of its analogs, truncated or substituted.

Unexpectedly, the results of the present invention showed that N-hexanoyl-, but not N-butyryl- or N-octanoyl-GRF(1-29)NH₂, statistically increased IGF-I levels when chronically administered in growing pigs. These results indicate that the addition of a C4 or a C8 chain at the N-terminus region of GRF yielded compounds with a poor biological activity when compared to the N-hexanoyl-GRF (C6-GRF). Therefore, the present invention teaches that the optimal length of the carbon chain to anchor to GRF to increase its bioactivity is C5 to C7. This result was unexpected based on the studies published by Coy et al., that demonstrated that the N-acetylation of GRF (addition of a C2 chain) increased its bioactivity in rats, and that did not document the activity of compounds with a carbon chain longer than C2.

According to the method of the present invention, these analogs can be produced either by anchoring one or several hydrophobic tails at the N- or C-terminal portion of GRF or its analogs, or by incorporating one or several pseudomicellar residues at any step of the chemical synthesis of GRF or its analogs. After cleavage and purification, the resulting modified peptide exhibits an enhanced biological activity when administered at very low dosage.

In accordance with the present invention, there is provided a chimeric fatty body-GRF analog with increased biological potency, of the following general formula:

Al-A2-Asp-Ala-Ile-Phe-Thr-A8-Ser-Tyr-Arg-Lys-
Val-Leu-Al5-Gln-Leu-Al8-Ala-Arg-Lys-Leu-Leu-
A24-Asp-Ile-A27-A28-Arg-A30

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wherein,

A2 is Val or Ala;

A15 is Ala or Gly;

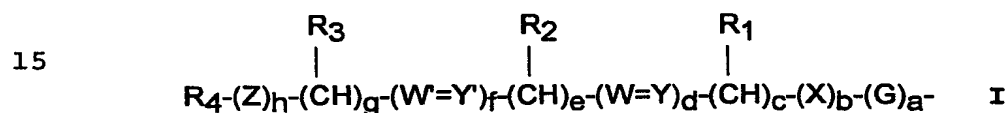
A24 is Gln or His;

5 A27 is Met, Ile or Nle;

A28 is Ser or Asp;

A30 is any amino alkyl carboxamide-NH-(CH₂)_n-CONH-, with n=1 to 12; or any amino acid sequence of 1 to 15 residues; and

10 A1 is Tyr or His; A8 is Asn or Ser and A18 is Ser or Thr, wherein A1 is N- or O-anchored by a hydrophobic tail of the following general formula I:



wherein,

G is a carbonyl, a phosphonyl, a sulfonyl or a sulfinyl group, with a= 0 or 1;

20 X is an oxygen atom, sulfur or an amino group (-NH-), with b= 0 or 1;

R₁, R₂ and R₃ radicals are identical or different, and are selected from a hydroxyl group, a hydrogen atom, and a lower linear or branched alkyl group;

25 -(W=Y)- and -(W'=Y')- represent the cis or trans double bounds -(CH=CR₅)- and -(CH=CR₆)- with R₅ and R₆= H or C₁-C₄ alkyl; with d and f= 0 or 1;

30 R₄ is an hydroxyl group, a hydrogen atom or a C₅-C₉ alkyl; and

Z is an oxygen or a sulfur atom; with h= 0 to 1 with the proviso that a, b, c, d, e, f, g and h

are identical or different and they are not all zero

35 when R₄ is a hydrogen, and the sum a, b, c, d, e, f, g and h is such that the hydrophobic tail of formula I

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has a linear main chain of between 5 and 8 atoms (C, O and/or S).

The preferred chimeric fatty body-pro-GRF analog of the present invention is selected from the group consisting of:

- a) wherein A1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein both a and b= 1; each of d, f and h= 0; G= carbonyl; X= oxygen atom; R₁, R₂, R₃, R₄= hydrogen atom and the sum c + e + g= 3, 4, 5 or 6;
- b) wherein A1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein a= 1; each of b, d, f and h= 0; G= carbonyl; R₁, R₂, R₃ and R₄= hydroxyl group and the sum c + e + g= 4, 5, 6 or 7;
- c) wherein A1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein a= 1; each of b and h= 0; the sum d + f= 1; G= carbonyl; R₁, R₂, R₃ and R₄= hydrogen atom and the sum c + e + g= 2, 3, 4 or 5; and
- d) wherein A1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein a= 1; each of b and h= 0; the sum d + f= 2; G= carbonyl; R₁, R₂, R₃ and R₄= hydrogen atom and the sum c + e + g= 0, 1, 2 or 3; and
- e) wherein A1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein a= 1; each of b, h, d and f= 0; G= carbonyl; R₁, R₂, R₃ and R₄= hydrogen atom; and the sum c + e + g= 4, 5, 6 or 7.

For the purpose of the present invention, the term "hydrophobic tail" or "Ht" is intended to mean any functionalized fatty body, such as fatty acids, fatty amines, fatty alcohols, cholesterol derivatives, etc.

The term "pseudomicellar residue" or "Pr" is intended

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to mean any α amino acid with side chain designed so that the residue may form or adopt a micellar structure in its switterionic form.

5 In accordance with the present invention, there is provided a pharmaceutical formulation for inducing growth hormone release which comprises as an active ingredient a GRF analog of the present invention in association with a pharmaceutically acceptable carrier, excipient or diluent.

10 In accordance with the present invention, there is provided a method of increasing the level of growth hormone in a patient which comprises administering to said patient an effective amount of a GRF analog of the present invention.

15 In accordance with the present invention, there is provided a method for the diagnosis of growth hormone deficiencies in patients, which comprises administering to said patient a GRF analog of the present invention and measuring the growth hormone response.

20 In accordance with the present invention, there is provided a method for the treatment of pituitary dwarfism or growth retardation in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

25 In accordance with the present invention, there is provided a method for the treatment of wound or bone healing in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

30 In accordance with the present invention, there is provided a method for the treatment of osteoporosis in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

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In accordance with the present invention, there is provided a method for improving protein anabolism (including protein sparing effect) in human or animal, which comprises administering to said human or animal
5 an effective amount of a GRF analog of the present invention.

In the present invention the amino acids are identified by the conventional three-letter abbreviations as indicated below, which are as generally
10 accepted in the peptide art as recommended by the IUPAC-IUB commission in biochemical nomenclature:

Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic Acid	Asp
Cysteine	Cys
Glutamic Acid	Glu
Glycine	Gly
Histidine	His
Leucine	Leu
Lysine	Lys
Methionine	Met
Ornithine	Orn
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophane	Trp
Tyrosine	Tyr
D-Tyrosine	tyr
Valine	Val

The term "natural amino acid" means an amino acid which occurs in nature or which is incorporated as an amino acid residue in a naturally occurring peptide.

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In addition, the abbreviation Nle is intended to mean Norleucine.

Other abbreviations used are:

- | | | |
|----|------------------|--|
| | TFA | Trifluoroacetic acid; |
| 5 | HOBt | 1-Hydroxybenzotriazole; |
| | DIC | Diisopropylcarbodiimide; |
| | DMF | Dimethylformamide; |
| | Pip | Piperidine; |
| | DMAP | 4-dimethylaminopyridine; |
| 10 | Boc | t-butyloxycarbonyl; |
| | Fmoc | Fluorenylmethyloxycarbonyl; |
| | BOP | Benzotriazo-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate; |
| | Me | Methyl; |
| 15 | HF | Hydrofluoric acid; |
| | NEt ₃ | Triethylamine; and |
| | TEAP | Triethylammonium phosphate (buffer). |

All the peptide sequences set out herein are written according to the generally accepted convention whereby the N-terminal amino acid is on the left and the C-terminal amino acid is on the right.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph of the effect of subcutaneously injected hGRF(1-29) NH₂ analogs on pig serum IGF-1;

Fig. 2 is a curve of the effect of one intravenous injection of (4µg/kg) hGRF(1-29) NH₂ and (4µg/kg) (Hexenoyl trans-3)₀ hGRF (1-29) NH₂ (TT-01024) +analog on pig serum GH;

Fig. 3 is a graph showing the effect of various doses of hGRF(1-29)NH₂ vs [hexenoyl trans-3]₀ hGRF(1-29)NH₂ (TT-01024) on the GH area under the curve over 300 minutes following I.V. administration

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(**P<0.01 and ***P<0.001 when compared to the basal period --60 to 0 min-);

Fig. 4 is a curve of the effect of one subcutaneous injection of 5µg/kg hGRF(1-29) NH₂ and (5µg/kg) (Hexenoyl trans-3)₀ hGRF (1-29) NH₂ analog on pig serum GH; and

Fig. 5 is a graph showing the effect of various doses of hGRF(1-29)NH₂ vs [Hexenoyl trans-3]₀ hGRF(1-29)NH₂ (TT-01024) on the GH area under the curve over 420 minutes following S.C. administration (**P<0.01 and ***P<0.001 when compared to the basal period --60 to 0 min-).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of fatty bodies, namely pseudomicellar residues and/or hydrophobic tails to produce a new family of highly potent, chimeric fatty body-pro-GRF analogs while remaining biodegradable and non immunogenic.

In accordance with the present invention, the fatty body-pro-GRF analogs can be chemically synthesized:

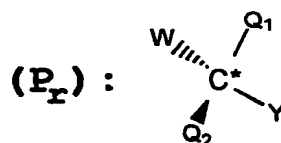
- by anchoring one or several hydrophobic tails at the C- and/or the N- terminal portion of GRF or one of its analogs, or
- by incorporating one or several pseudomicellar α amino acid derivative(s) ("pseudomicellar residue") in the chemical synthesis of GRF or one of its analogs.

In accordance with the present invention, the structure of pseudomicellar residues (Pr) used as a link in the synthesis of GRF and analogs thereof may be represented in the following manner:

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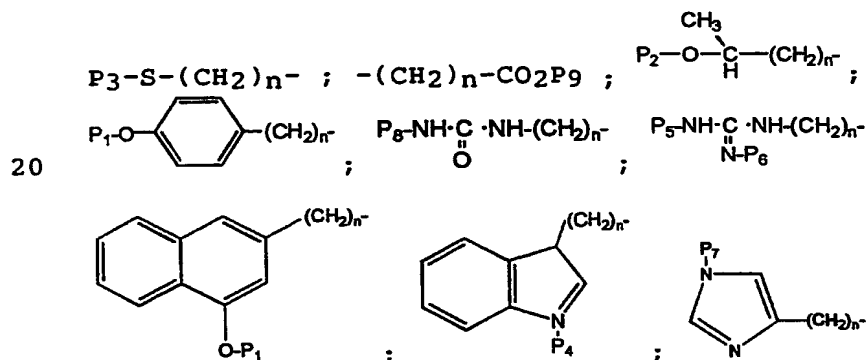
wherein:

W is a group selected from the group consisting of -CO₂Q₃; -PO₃Q₃ and -SO₃Q₃;

5 Q₃ is an hydrogen atom, an ammonium ion, an element selected from the group consisting the elements of group 1A of the Mendeléeve periodical table, or a functional group derived from the following fatty bodies, pentenoic acids, hexenoic acids, heptenoic
10 acids or their saturated forms;

Q₁ is a radical selected from the group consisting of alkenyl, aralkyl, aryl and alkyl (C_{n1}H_{2n1+1}), where n₁ is a number between 1 and 8. Q₁ may be selected from the following list, which is provided to illustrate the invention rather than to limit
15 its scope:

P₂-O-(CH₂)_n- ; P₈-NH-CO-(CH₂)_n- ; P₄-NH-(CH₂)_n- ;



wherein,

25 P₁ to P₉ represents a hydrogen atom; a methyl group; a functional hydrophobic tail with a main aliphatic, alicyclic or aromatic chain, linear or branched which may be selected from the following list: saturated fatty acid of general formula (C_mH_{2m}O₂) with m

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being a value between 4 and 12; or a lateral chain protecting group as described by Gross et Meienhofer (1981, *The peptides*, vol. 3, Academic press: pages 1-341) such that P₁ may be a benzyl group, bromo-2 benzyl, dichloro-2,6 benzyl or t-butyl; P₂ may be a benzyl group or t-butyl; P₃ may be a benzyl group, t-butyl, trityl, acetamidomethyl or benzamidomethyl; P₄ may be trifluoroacetyl, t-butyloxycarbonyl (Boc), benzyloxycarbonyl (Z) or fluorenylmethyloxycarbonyl (Fmoc); P₅ may be a nitro group, p-methoxybenzenesulfonyl, mesitylenesulfonyl, or pentamethylcromane; with the proviso that P₆ is hydrogen, or that P₅ and P₆ may be adamantyloxycarbonyl; P₇ may be a phenacyl group, benzyloxymethyl or t-butoxymethyl; P₈ may be a benzhydryl group, dimethoxybenzhydryl, trityl or xanthenyl;

n is an integer between 0 and 6;

Y is of the following general formula :



wherein:

A is a bivalent heteroatom, preferably oxygen, sulfur, a -NH- group or a -N(Me)- group;

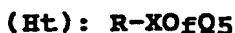
P_Z is the same as P₁ to P₄ defined previously where Z is an integer between 1 to 4; and

Q₂ is an hydrogen atom. When Q₁=H or lower alkyl, Q₂ may be any alkyl, alkoxy, alkenyl, aralkyl, or aryl group. In these conditions it possesses the same chemical identity as defined above for Q₁.

The carbon atoms on which (Q₁) and (Q₂) are attached are of L or D configuration. They are asymmetrical but not when (Q₁)=(Q₂) or (W)=(Y).

In cases where the anchoring consists in one or more hydrophobic tails (Ht) non-pseudomicellar, the whole of the structure of said tails may be represented as follows:

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wherein:

R is an alkyl, alkenyl, aryl or aralkyl radical of branched or linear chains, and may be derived from the group of metabolisable fatty bodies consisting of
5 saturated fatty acids of the general formula $C_mH_{2m}O_2$, preferably with m being an integer between 4 and 6; mono or polyunsaturated fatty acids, fatty amines and alcohols;

10 X represent a phosphorous, a carbon or a sulfur atom;

f is an integer between 1 and 3;

Q5 represent an hydrogen atom, an ammonium ion, or an alkaline metal ion; when f is an integer between 1 and 2, Q5 may be defined as for R above with the pro-
15 viso of having at least one of the following functions:

amino (-NH-); alcohol(-OH), thio (-SH), or acid(-XO_fH); with X and f being as defined above.

For a better carrying out of the chemical anchoring reaction, hydrophobic tails or pseudomicellar
20 residues functionalized under the acid form are preferably used. In these conditions, the anchoring reaction is preferably effected in a solid phase (Merrifield R.B., 1963, *J. Am. Chem. Soc.*, 85:2149; 1964, *J. Am. Chem. Soc.*, 86:304) using extremely active
25 reagents such as for example Benzotriazole-1-yloxytris (dimethylamino) phosphonium hexafluoro-phosphate known in the prior art (B. Castro et al., 1975, *Tetrahedron letters*, Vol. 14:1219).

The pseudomicellar residue to be anchored is
30 generally prepared by the direct action of a malonic salt, preferably a sodium salt of diethylacetamidomethyl malonate, and the alkyl, alkenyl, aryl or aralkyl halide in a polar solvent such as dimethylformamide. This reaction is usually followed by an acid or

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alkaline hydrolysis and of a resolution (preferably enzymatic) of the resulting racemic mixture.

In certain conditions, the preparation of the pseudomicellar residue consists in:

- 5 a) a first step; to protect in an orthogonal fashion and to attached on a solid support of sarsin type (M. Merçler et al., 1988, *Peptides, Chemistry and Biology*, Proceedings of the 10th American peptide symposium, St. Louis, p.259, G.R. Marshall, Ed., Escom, leiden), an amino
10 acid with a functionalized lateral chain such as lysine, glutamic acid or aspartic acid; and
- 15 b) a second step; to specifically deprotect the lateral chain and to anchor on the free site a metabolisable hydrophobic tail (Ht) such as
described above. The pseudomicellar residue (Pr) is thus obtained after a cleavage (0.5% TFA/CH₂Cl₂) of the support-residue bond, followed by purification steps.

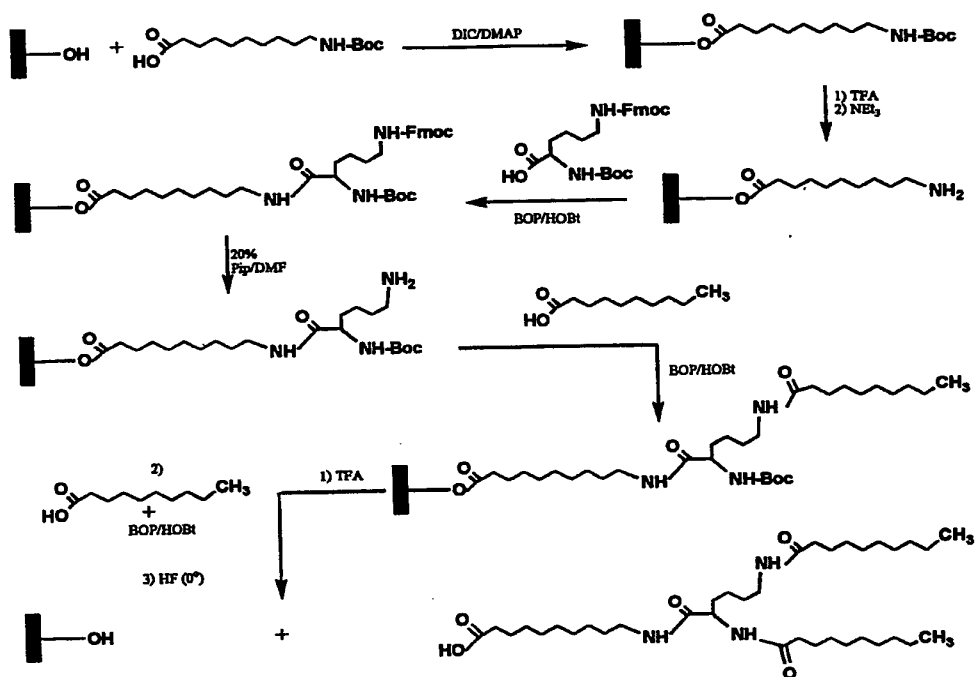
- 20 The pseudomicellar residue may also be prepared by a selective complexion of the acid and the amine function in alpha of a trifunctional free amino acid, by complexing agents of mineral origin such as copper acetate. In these conditions, the anchoring of the
25 metabolisable hydrophobic tail is effected by the direct action of the formed complex and of said tail, either in its acyl halide form or in its acid or amine form in the presence of a condensation agent.

- 30 In the case where the hydrophobic tail to be anchored consists in a fatty acid, the activation in view of the anchoring may be carried out *in situ*. Depending on the synthesis strategies used, the peptide anchoring site is liberated just prior to the anchoring in traditional deprotection conditions (Gross et
35 Meienhofer, 1981, *The peptides*, vol. 3, Academic press:

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General GRF analogs synthesis steps were carried out by solid-phase methodology on a 9050™ plus

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peptide synthesizer (Millipore Corporation, Milford, MA) using Fmoc strategy and synthesis cycles supplied by Millipore. Fmoc amino acids were supplied by Bachem California and other commercial sources. Sequential Fmoc chemistry using BOP/HOBt as coupling methodology were applied to the starting Fmoc-Pal-PEG resin (Millipore, catalog number: GEN 913383) for the production of C-terminal carboxamides. Fmoc deprotections were accomplished with piperidine 20% solution in DMF. After synthesis completion, the resin was well washed with DMF and ether prior to drying. Final cleavages of side chain protecting groups and peptide-resin bonds were performed using Millipore supplied procedure consisting of the following mixture: TFA, water, phenol, triisopropylsilane (88:5:5:2). Peptides were then precipitated and washed with ether prior to drying. Reverse phase HPLC purification (buffer A: TEAP 2.5; buffer B: 80% CH₃CN in A) using a water pep 4000, absorbance 214nm, detector model 486, flow rate 50ml/min.; linear gradient generally from 25 to 60%B in 105 min.) followed by a desalting step (buffer C: 0.1% TFA in H₂O; buffer D: 0.1% TFA in CH₃CH/H₂O 80:20) afforded peptides in yields amounting from 10 to 30% with homogeneity greater than 97% as estimated by HPLC (millennium/photodiode array detection).

In accordance with the present invention, pig was selected as a test specie, since it is a valuable preclinical model for the development of GRF analogs. Indeed, human and porcine GRF(1-29)NH₂ share a 100% homology of structure, and the physiological pattern of GH secretion is almost identical in both species.

Moreover, the potency of the GRF analogs was assessed as their ability to significantly increase IGF-I blood levels rather than their acute GH releasing potency. Indeed, it is known that the anabolic and

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healing effects of GH or GRF induced GH are mediated by an increase in IGF-I synthesis and secretion. Therefore, the measurement of GRF induced IGF-I elevation is the best indicator of the treatment efficacy.

5 The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

10

EFFECT OF REPEATED ADMINISTRATIONS OF
[BUTYRYL⁰], [OCTANOYL⁰]-, [HEXANOYL⁰]- [HEXANOYL³⁰],
[HEXANOYL^{0,30}], HGRF(1-29)NH₂ AND [HEXANOYL⁰]
HGRF(1-44)NH₂ VS HGRF(1-29)NH₂
ON SERUM IGF-I LEVELS IN PIGS

15

The objective of these experiments was to assess the potential of the GRF analogs as anabolic agents. It is known that GH or GRF-induced GH secretion exert their anabolic effect via an increase in
20 insulin-like growth factor I (IGF-I) synthesis and secretion, that result in elevated levels of circulating IGF-I. It has been previously demonstrated that the intensity of the anabolic response to a GRF analog treatment is proportional to the increase in IGF-I
25 levels in pigs (Dubreuil P. et al., 1990, J. Anim. Sci., 68:1254-1268).

Therefore, in order to investigate the anabolic potency of the fatty acid-pro-GRF analogs, their ability to increase IGF-I levels following repeated
30 S.C. administrations in pig was evaluated.

Experiment 1

26 Landrace x Yorkshire castrated male pigs (40-45kg BW) were randomly distributed into 4 experimental groups:
35

- 1- hGRF(1-29)NH₂ (20µg/kg, n=7)
- 2- [octanoyl⁰] hGRF(1-29)NH₂ (20µg/kg, n=6)
- 3- [hexanoyl⁰] hGRF(1-29)NH₂ (20µg/kg, n=6)

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4- [butyryl⁰] hGRF(1-29)NH₂ (20µg/kg, n=7)

Each animal was injected BID (twice a day) subcutaneously for 4 consecutive days. One blood sample was collected each morning prior to the first injection of the day, and the day after the last injection, for IGF-I measurement.

Experiment 2

40 Landrace x Yorkshire castrated male pigs (40-45 kg BW) were randomly distributed into 5 experimental groups:

- 1- saline (n=8)
- 2- hGRF(1-29)NH₂ (40µg/kg, n=8)
- 3- [hexanoyl⁰] hGRF(1-29)NH₂ (10µg/kg, n=8)
- 4- [hexanoyl⁰] hGRF(1-29)NH₂ (20µg/kg, n=8)
- 15 5- [hexanoyl⁰] hGRF(1-29)NH₂ (40µg/kg, n=8)

Each animal was injected BID (twice a day) subcutaneously for 5 consecutive days. One blood sample was collected each morning prior to the first injection of the day, and the day after the last injection, for IGF-I measurement.

Experiment 3:

48 Landrace x Yorkshire castrated male pigs (40-45 kg BW) were randomly distributed into 6 experimental groups:

- 1- Saline (n=8)
- 2- hGRF(1-44)NH₂ (30µg/kg, n=8)
- 3- [hexanoyl⁰]hGRF(1-44)NH₂ (30µg/kg, n=8)
- 4- [hexanoyl⁰]hGRF(1-29)NH₂ (20µg/kg, n=8)
- 30 5- [hexanoyl³⁰]hGRF(1-29)NH₂ (20µg/kg, n=8)
- 6- [hexanoyl^{0, 30}]hGRF(1-29)NH₂ (20µg/kg, n=8)

The selected doses were 30µg/kg for hGRF(1-44)NH₂ analogs and 20µg/kg for hGRF(1-29)NH₂ analogs, which give identical doses on a molar basis. Each animal was injected BID (twice a day) subcutaneously for 5 consecutive days. One blood sample was collected each

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morning prior to the first injection of the day, and the day after the last injection, for IGF-I measurements.

5 IGF-I measurements

IGF-I levels were measured in pig serum by double antibody radioimmunoassay after formic acid-acetone extraction, as previously described (Abribat T. et al., 1993, J. Endocrinol., 39:583-589). The extraction
10 prior to radioimmunoassay is a necessary step to remove endogenous IGF-binding proteins.

Statistical analysis

In both experiments, the IGF-I data were ana-
15 lyzed by a two way repeated measure analysis of variance, with day and treatment (GRF analog) as sources of variation. Multiple comparison procedures were there run (Student-Newman Keuls method). A $P < 0.05$ was con-
sidered as statistically significant.

20

Results

Experiment 1

There were both a significant effect of day
($P=0.0004$) and a significant treatment x day interac-
25 tion ($P=0.011$), indicating that the increase in IGF-I levels was dependent on the analog tested (Table 1). Blood samples for IGF-I measurements were collected daily prior to the first injection of compounds. Data are shown as mean \pm SEM of 6 to 7 values per group.

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Table 1

**Effect of repeated SC injection (20µg/kg BID x 4 days)
of GRF analogs on serum IGF-I levels**

5

Treatment (BID, 20µg/kg SC)	Day 1 (pretreatment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)
hGRF(1-29)NH ₂	252 ± 28	235 ± 19	263 ± 16	258 ± 17	262 ± 24
[octanoyl ⁰] hGRF(1-29)NH ₂	316 ± 22	287 ± 20	301 ± 37	301 ± 37	318 ± 39
[hexanoyl ⁰] hGRF(1-29)NH ₂	248 ± 20	281 ± 28	299 ± 26	319 ± 22 ^a	342 ± 21 ^{a,b}
[butyryl ⁰] hGRF(1-29)NH ₂	278 ± 20	281 ± 24	302 ± 26	289 ± 26	293 ± 23

Treatment P=0.42

Day P=0.0004

Treatment x Day P=0.011

^a P < 0.05 when compared to day 110 ^b P < 0.05 when compared to day 2

Multiple comparisons revealed that only [hexanoyl⁰] hGRF(1-29)NH₂ elicited an increase in IGF-I levels, which was significant on days 4 (29%, P < 0.05) and 5 (38%, P < 0.05). Human GRF(1-29)NH₂ had no effect

15 on IGF-I levels at the dose tested.

Experiment 2

There were both a significant effect of day (P < 0.0001) and a significant treatment x day inter-

20 action (P < 0.0001), indicating that the increase in IGF-I levels was dependent on the analog tested (Table 2). Blood samples for IGF-I measurements were collected daily prior to the first injection of the day. Data are shown as mean ± SEM of 8 values per

25 group.

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Table 2

Dose-related effect of repeated SC injection (BID x 5 days) of GRF analogs on serum IGF-I levels

Treatment BID, SC	Day 1 (pretreat- ment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)	Day 6 (ng/ml)
saline	282 ± 33	266 ± 30	281 ± 34	293 ± 30	287 ± 32	289 ± 33
hGRF(1-29)NH ₂ (40µg/kg)	244 ± 24	243 ± 16	267 ± 20	275 ± 27	267 ± 17	256 ± 15
[hexanoyl ⁰] hGRF (1-29)NH ₂ (10µg/kg)	303 ± 31	327 ± 20	337 ± 25	338 ± 25	366 ± 37 ^a	350 ± 34 ^a
[hexanoyl ⁰] hGRF (1-29)NH ₂ (20µg/kg)	302 ± 38	341 ± 37	368 ± 43 ^a	362 ± 40 ^a	362 ± 45 ^a	368 ± 57 ^a
[hexanoyl ⁰] hGRF (1-29)NH ₂ (40µg/kg)	252 ± 35	275 ± 32	319 ± 31 ^a	354 ± 41 ^{a,b}	350 ± 34 ^{a,b}	374 ± 33 ^{a,b,c}

5 Treatment P=0.23; Day P=0.0001

Treatment x Day P=0.0001

^a P < 0.05 when compared to day 1

^b P < 0.05 when compared to day 2

^c P < 0.05 when compared to day 3

10

Multiple comparisons revealed that all three tested doses of [hexanoyl⁰] hGRF(1-29)NH₂ increased IGF-I levels. At 10µg/kg, IGF-I levels were significantly increased at days 5 and 6 (16 to 21%, P < 0.05). At 20µg/kg, they were increased at days 3, 4, 5 and 6 (20 to 22%, P < 0.05). At 40µg/kg, they were increased at days 3, 4, 5 and 6 (27 to 48%, P < 0.05). The serum IGF-I levels remained stable in saline - and hGRF(1-29)NH₂ - treated pigs.

20

Finally, a regression analysis revealed that the increase in IGF-I concentrations from day 1 to day 6 was dependent on the dose of [hexanoyl⁰] hGRF(1-29)NH₂ ($\Delta\text{IGF-I} = 11.9 + (2.77 * \text{dose})$; $r = 0.68$, P < 0.0001).

25

Experiment 3: There were both a significant effect of day (P<0.0001) and a significant treatment x day interaction (P<0.0001), indicating that the increase in IGF-

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I levels was dependent on the analog tested (Table IV). Multiple comparison revealed that analogs with an hexanoyl function branched at the N-terminal region of GRF were highly potent:

5 - [hexanoyl⁰] hGRF(1-29)NH₂ significantly increased IGF-I levels on days 5 and 6 (by 28% and 31%, P<0.05)

- [hexanoyl⁰, 30] hGRF(1-29)NH₂ significantly increased IGF-I levels on days 4, 5 and 6 (by 32%, 35% and 43%, P<0.05)

10 - [hexanoyl⁰] hGRF(1-44)NH₂ significantly increased IGF-I levels on days 3, 4, 5 and 6 (by 41%, 54%, 50% and 61%, P<0.05)

As previously observed for hGRF(1-29)NH₂ (experiments 1 and 2), the full length hGRF(1-44)NH₂ had little or no effect on IGF-I levels (except for a significant effect on day 5, which was not sustained on day 6). Finally, the anchoring of an hexanoyl function at the C-terminal region of hGRF(1-29)NH₂ yielded an analog with increased potency when compared to hGRF(1-29)NH₂ (21% increased in IGF-I levels on day 6, P<0.05), but less potent than [hexanoyl⁰]hGRF(1-29)NH₂.

Human GRF(1-29)NH₂ and hGRF(1-44)NH₂ were injected at 20µg/kg and 30µg/kg, respectively, in order to achieve equimolar concentrations. Data shown are mean ± SEM of 8 values per group.

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Table 3

Effect of multiple SC injections of GRF analogs (BID x 5 days) on serum IGF-I levels in growing pigs

Treatment BID, SC	Day 1 (pretreat- ment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)	Day 6 (ng/ml)
saline	215 ± 21	215 ± 28	219 ± 25	226 ± 28	249 ± 30	234 ± 24
hGRF(1-44)NH ₂ (30 µg/kg)	245 ± 21	254 ± 22	285 ± 26	297 ± 28	303 ± 26 ^a	296 ± 26
[hexanoyl ⁰] hGRF(1- 29)NH ₂ (20 µg/kg)	272 ± 45	292 ± 52	292 ± 57	315 ± 57	347 ± 44 ^{a,b,c}	356 ± 44 ^{a,b,c}
[hexanoyl ³⁰] hGRF(1- 29)NH ₂ (20 µg/kg)	297 ± 30	270 ± 25	287 ± 24	278 ± 18	276 ± 20	327 ± 24 ^b
[hexanoyl ^{0,30}] hGRF(1- 29)NH ₂ (20 µg/kg)	205 ± 24	212 ± 26	253 ± 33	271 ± 36 ^{a,b}	277 ± 29 ^{a,b}	294 ± 26 ^{a,b}
[hexanoyl ⁰] hGRF(1- 44)NH ₂ (30 µg/kg)	241 ± 30	290 ± 33	340 ± 41 ^a	372 ± 40 ^{a,b}	361 ± 46 ^{a,b}	388 ± 49 ^{a,b,c}

5 Treatment P=0.16
Day P<0.0001
Treatment x Day P<0.0001

^a P < 0.05 when compared to day 1
^b P < 0.05 when compared to day 2
^c P < 0.05 when compared to day 3

Conclusions

10 Neither hGRF(1-29)NH₂ nor hGRF(1-44)NH₂ at
doses ranging from 20 to 40 µg/kg were able to modulate
IGF-I levels. However, the anchoring of fatty acid
rendered GRF more potent and yielded analogs with mark-
15 edly improved activity on IGF-I secretion. The anchor-
ing of fatty acids was efficient in improving the ana-
bolic potency of both hGRF(1-29)NH₂ and hGRF(1-44)NH₂.
From the above results, it is concluded that the ideal
fatty acid to use is hexanoic acid or any C6 fatty
20 derivative, and that it should be preferably anchored
at the N-terminal region of GRF to yield maximally
potent analogs.

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EXAMPLE II**Comparative effects of pro-GRF analogs on IGF-I levels in pigs**

5 This was a 5-day treatment, twice a day S.C. administration of one single dose of each test article vs saline. This experiment was conducted to compare the efficacy of (Aminohexanoyl)₀ hGRF (1-29) NH₂, (Hexylformiate)₀ hGRF (1-29) NH₂, (Hexenoyl trans-2)₀ hGRF (1-29) NH₂, (Hexenoyl trans-3)₀ hGRF (1-29) NH₂ and (Muconoyl)₀ hGRF (1-29) NH₂ to that of (Hexanoyl)₀ hGRF (1-29) NH₂.

15 All tested compounds belong to the same family of GRF analogs: they are a combination of the natural GRF and natural fatty acids, designed to improve the activity of the molecule.

Identity of tested analogs:

		<u>in saline</u>
20	TT-01015 (Hexanoyl) ₀ hGRF (1-29) NH ₂	20 µg/kg
	TT-01021 (Aminohexanoyl) ₀ hGRF (1-29) NH ₂	20 µg/kg
	TT-01022 (Hexylformiate) ₀ hGRF (1-29) NH ₂	20 µg/kg
	TT-01023 (Hexenoyl trans-2) ₀ hGRF (1-29) NH ₂	20 µg/kg
	TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	20 µg/kg
25	TT-01025 (Muconoyl) ₀ hGRF (1-29) NH ₂	20 µg/kg

Route and frequency of test article

ADMINISTRATION: Two daily subcutaneous injections.

TEST SYSTEM: Landrace x Yorkshire pigs.

30 ANIMAL DESCRIPTION: Fifty six (56) growing barrows pigs weighing 35 kg at the time of purchase.

RATION: Commercial feed concentrate (18% protein) offered ad libitum.

EXPERIMENTAL

35 DESIGN: Fifty six (56) pigs were randomly distributed into 7 experimental groups (n = 8 pigs per group). Each group received two

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daily S.C. administration of the following
treatments (volume: 3 ml, S.C. injection).

- group 1: saline 2 x/day
group 2: TT-01015 20 µg/kg 2 x/day
5 group 3: TT-01021 20 µg/kg 2 x/day
group 4: TT-01022 20 µg/kg 2 x/day
group 5: TT-01023 20 µg/kg 2 x/day
group 6: TT-01024 20 µg/kg 2 x/day
10 group 7: TT-01025 20 µg/kg 2 x/day

Treatments were administered from day 1 to 5.
Immediately before the injections, one blood sample
were collected from each animal, and additional blood
samples were collected on day 6.

- 15 Blood samples were allowed to clot, serum was
harvested by centrifugation and submitted to IGF-I
assays.

- Results are shown in Fig. 1 as D-IGF-I, which
is defined as the increase in IGF-I levels from day 1
20 (pretreatment levels) to day 6 (after 5 days of GRFs
administrations). Among all analog tested, only
hexanoyl-, hexylformiate-, hexenoyl trans2- and
hexenoyl trans3-hGRF(1-29)NH₂ significantly increased
IGF-I levels over the 6-day study period, whereas ami-
25 nohexanoyl- and muconoyl-hGRF(1-29)NH₂ did not. Since
hGRF(1-29)NH₂ has been shown to be ineffective at the
same dose in the same conditions in previous assays
(see Example I), these results show that the addition
of various C6 carbon chains at the N-terminus region of
30 GRF increases its bioactivity.

EXAMPLE III

- 35 Intravenous GH-releasing potency of (Hexenoyl trans-3)₀
hGRF (1-29) NH₂ vs hGRF(1-29)NH₂ in pigs

This experiment was conducted to test the I.V.
acute GH-releasing potency of (Hexenoyl trans-3)₀ hGRF

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(1-29) NH₂, a pro-GRF analog, in a model physiologically close to human and to compare it to that of hGRF(1-29)NH₂.

(Hexenoyl trans-3)₀ hGRF (1-29) NH₂ is a combination of the natural hGRF(1-29)NH₂ and natural fatty acids. This study was a multidose, single I.V. injection study.

Identity of tested analogs:

10

TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	0.25 µg/kg
TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	1 µg/kg
TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	4 µg/kg
hGRF(1-29)NH ₂	0.25 µg/kg
15 hGRF(1-29)NH ₂	1 µg/kg
hGRF(1-29)NH ₂	4 µg/kg

Route and frequency of test article

ADMINISTRATION: intravenous acute injection.

TEST SYSTEM: Landrace x Yorkshire pigs.

20 ANIMAL DESCRIPTION: Fifty six (56) growing barrows pigs weighing 35 kg at the time of purchase.

RATION: Commercial feed concentrate (18% protein) offered ad libitum.

25 EXPERIMENTAL

DESIGN: Fifty (56) pigs (4 spare animals) were cannulated (a catheter surgically implanted in one jugular vein) within one week, before the study. On days 1 and 7, cannulated animals were randomly distributed into 7 groups (n = 4 pigs per group).

30

group 1: saline

group 2: TT-01024 0.25 µg/kg

group 3: TT-01024 1 µg/kg

35 group 4: TT-01024 4 µg/kg

group 5: hGRF(1-29)NH₂ 0.25 µg/kg

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group 6: hGRF(1-29)NH₂ 1 µg/kg
group 7: hGRF(1-29)NH₂ 4 µg/kg

Blood samples for pGH assay were collected every 20 min from 1 hour before to 5 hours after GRF injections, with additional samplings 10 and 30 min after injection (n = 21 samples). Blood samples are allowed to clot at +4°C. Serum will be harvested by centrifugation, stored at -20°C and submitted to pGH assays.

Results are illustrated in Figs. 2 and 3. As shown in Fig. 2, hGRF(1-29)NH₂ (4 µg/kg) induced a rapid GH release that was sustained for approximately 60 minutes following injection. In contrast, hexenoyl trans3-hGRF(1-29)NH₂ injected at the same dose increased GH levels over a longer period, approximately 260 minutes. In addition, the GH response in the first 60 minutes was moderate, suggesting that this analog acts as a pro-GRF, being processed in serum into native GRF in the minutes or hours following injection. As shown in Fig. 3, which presents the effects of various doses of GRF and the analog on the GH area under the curve (0 to 300 minutes following injection), hGRF(1-29)NH₂ produced a significant effect on GH secretion at 4µg/kg, but not at 0.25 or 1 µg/kg, whereas hexenoyl trans3-hGRF(1-29)NH₂ elicited a significant response at all 3 doses tested. In conclusion, these results show that hexenoyl trans3-hGRF(1-29)NH₂ is a GRF analog with increased potency on GH secretion, and suggest that it may act as a pro-GRF, being protected from enzymatic degradation in serum.

EXAMPLE IV

Subcutaneous GH-releasing potency of (Hexenoyl trans-3)₀ hGRF (1-29) NH₂ vs hGRF(1-29)NH₂ in pigs

This experiment was conducted to test the S.C. acute GH-releasing potency of (Hexenoyl trans-3)₀ hGRF

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(1-29) NH₂, a pro-GRF analog, in a model physiologically close to human and to compare it to that of hGRF(1-29)NH₂.

Identity of tested analogs:

5	TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	0.31 µg/kg
	TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	1.25 µg/kg
	TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	5 µg/kg
	TT-01024 (Hexenoyl trans-3) ₀ hGRF (1-29) NH ₂	20 µg/kg
	hGRF(1-29)NH ₂	1.25 µg/kg
10	hGRF(1-29)NH ₂	5 µg/kg
	hGRF(1-29)NH ₂	20 µg/kg

Route and frequency of test article

	ADMINISTRATION:	Subcutaneous acute injection.
15	TEST SYSTEM:	Landrace x Yorkshire pigs.
	ANIMAL DESCRIPTION:	Sixty four (64) growing barrows pigs weighing 35 kg at the time of purchase.
20	RATION:	Commercial feed concentrate (18% protein) offered ad libitum.

EXPERIMENTAL

25	DESIGN:	Thirty six (36) pigs (4 spare animals) were cannulated (a catheter surgically implanted in one jugular vein) within one week, before the study. On days 1 and 7, cannu- lated animals were randomly distributed into 8 groups (n = 4 pigs per group).
----	---------	--

	group 1:	saline	
	group 2:	TT-01024	0.31 µg/kg
30	group 3:	TT-01024	1.25 µg/kg
	group 4:	TT-01024	5 µg/kg
	group 5:	TT-01024	20 µg/kg
	group 6:	hGRF(1-29)NH ₂	1.25 µg/kg
	group 7:	hGRF(1-29)NH ₂	5 µg/kg
35	group 8:	hGRF(1-29)NH ₂	20 µg/kg

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Blood samples for pGH assay were collected every 20 min from 1 hour before to 7 hours after GRF injections, (n = 25 samples). Blood samples were allowed to clot at +4CC. Serum is harvested by centrifugation, stored at -20°C and submitted to pGH assays.

Results are shown in Figs. 4 and 5. As shown in Fig. 4, the subcutaneous injection of 5 µg/kg hGRF(1-29)NH₂ induced a GH response in the first 60 minutes following administration, whereas the same injection of hexenoyl trans3-hGRF(1-29)NH₂ induced a GH response that was sustained for 240 minutes. The Fig. 5 illustrates the effect of various doses of the GRFs tested on the GH area under the curve over the study period, i.e. from 0 to 420 minutes following injection. Over this period, hGRF(1-29)NH₂ did not induce any significant GH response at any of the tested doses, whereas hexenoyl trans3-hGRF(1-29)NH₂ elicited significant increases of the GH AUC at 5 and 20 µg/kg. Altogether, these results suggest that hexenoyl trans3-hGRF(1-29)NH₂ is a highly potent GH secretagogue, even when subcutaneously administered.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: THERATECHNOLOGIES INC.
- (B) STREET: 7701 - 17eme Avenue
- (C) CITY: Montreal
- (D) STATE: Quebec
- (E) COUNTRY: Canada
- (F) POSTAL CODE (ZIP): H2A 2S5
- (G) TELEPHONE: (514) 729-7904
- (H) TELEFAX: (514) 593-8142

(ii) TITLE OF INVENTION: CHIMERIC FATTY BODY-PRO-GRF ANALOGS
WITH INCREASED BIOLOGICAL POTENCY

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/453,067
- (B) FILING DATE: 26-MAY-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln
1				5					10					15	
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg	Gln	Gln	Gly
				20				25					30		
Glu	Ser	Asn	Gln	Glu	Arg	Gly	Ala	Arg	Ala	Arg	Leu				
				35				40							

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(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln
1				5					10					15	
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg			
			20					25							

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WE CLAIM:

1. A chimeric fatty body-pro-GRF analog with increased biological potency, of the following general formula:

A1-A2-Asp-Ala-Ile-Phe-Thr-A8-Ser-Tyr-Arg-Lys-
Val-Leu-A15-Gln-Leu-A18-Ala-Arg-Lys-Leu-Leu-
A24-Asp-Ile-A27-A28-Arg-A30

wherein,

A2 is Val or Ala;

A15 is Ala or Gly;

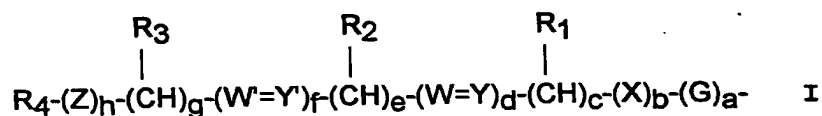
A24 is Gln or His;

A27 is Met, Ile or Nle;

A28 is Ser or Asp;

A30 is any amino alkyl carboxamide-NH-(CH₂)_n-CONH-, with n=1 to 12; or any amino acid sequence of 1 to 15 residues; and

A1 is Tyr or His; A8 is Asn or Ser and A18 is Ser or Thr, wherein A1 is N- or O-anchored by a hydrophobic tail of the following general formula I:



wherein,

G is a carbonyl, a phosphonyl, a sulfonyl or a sulfinyl group, with a= 0 or 1;

X is an oxygen atom, sulfur or an amino group (-NH-), with b= 0 or 1;

R₁, R₂ and R₃ radicals are identical or different, and are selected from a hydroxyl group, a hydrogen atom, and a lower linear or branched alkyl group;

-(W=Y)- and -(W'=Y')- represent the cis or trans double bounds -(CH=CR₅)- and -(CH=CR₆)-

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with R_5 and $R_6 = H$ or C_1-C_4 alkyl; with d and $f = 0$ or 1 ;

R_4 is an hydroxyl group, a hydrogen atom or a C_5-C_9 alkyl; and

Z is an oxygen or a sulfur atom; with $h = 0$ to 1 with the proviso that a, b, c, d, e, f, g and h are identical or different and they are not all zero when R_4 is a hydrogen, and the sum of a, b, c, d, e, f, g and h is such that the hydrophobic tail of formula I has a linear main chain of between 5 and 8 atoms (C, O and/or S).

2. The chimeric fatty body-pro-GRF analog of claim 1, wherein A_1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein both a and $b = 1$; each of d, f and $h = 0$; $G =$ carbonyl; $X =$ oxygen atom; $R_1, R_2, R_3, R_4 =$ hydrogen atom and the sum $c + e + g = 3, 4, 5$ or 6 .

3. The chimeric fatty body-pro-GRF analog of claim 1, wherein A_1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein $a = 1$; each of b, d, f and $h = 0$; $G =$ carbonyl; R_1, R_2, R_3 and $R_4 =$ hydroxyl group and the sum $c + e + g = 4, 5, 6$ or 7 .

4. The chimeric fatty body-pro-GRF analog of claim 1, wherein A_1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein $a = 1$; each of b and $h = 0$; the sum $d + f = 1$; $G =$ carbonyl; R_1, R_2, R_3 and $R_4 =$ hydrogen atom and the sum $c + e + g = 2, 3, 4$ or 5 .

5. The chimeric fatty body-pro-GRF analog of claim 1, wherein A_1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein $a = 1$; each of b and

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$h = 0$; the sum $d + f = 2$; $G = \text{carbonyl}$; R_1, R_2, R_3 and $R_4 = \text{hydrogen atom}$ and the sum $c + e + g = 0, 1, 2$ or 3 .

6. The chimeric fatty body-pro-GRF analog of claim 1, wherein A_1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein $a = 1$; each of b, h, d and $f = 0$; $G = \text{carbonyl}$; R_1, R_2, R_3 and $R_4 = \text{hydrogen atom}$; and the sum $c + e + g = 4, 5, 6$ or 7 .

7. A pharmaceutical formulation for inducing growth hormone release which comprises as an active ingredient a GRF analog as claimed in claim 1, 2, 3, 4 or 5, in association with a pharmaceutically acceptable carrier, excipient or diluent.

8. A method of increasing the level of growth hormone in a patient which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1, 2, 3, 4 or 5.

9. A method for the diagnosis of growth hormone deficiencies in patients, which comprises administering to said patient a GRF analog as claimed in claim 1, 2, 3, 4 or 5 and measuring the growth hormone response.

10. A method for the treatment of pituitary dwarfism or growth retardation in a patient, which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1, 2, 3, 4 or 5.

11. A method for the treatment of wound or bone healing in a patient, which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1, 2, 3, 4 or 5.

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12. A method for the treatment of osteoporosis in a patient, which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1, 2, 3, 4 or 5.

13. A method for improving protein anabolism in human or animal, which comprises administering to said human or animal an effective amount of a GRF analog as claimed in claim 1, 2, 3, 4 or 5.

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AMENDED CLAIMS

[received by the International Bureau on 04 November 1996 (04.11.96);
original claims 1-13 replaced by new claims 1-25 (5 pages)]

1. A chimeric fatty body-pro-GRF analog with increased biological potency, of the following general formula:

Al-A2-Asp-Ala-Ile-Phe-Thr-A8-Ser-Tyr-Arg-Lys-
Val-Leu-Al5-Gln-Leu-Al8-Ala-Arg-Lys-Leu-Leu-
A24-Asp-Ile-A27-A28-Arg-A30-R₀

wherein,

Al is Tyr or His;

A2 is Val or Ala;

A8 is Asn or Ser;

Al5 is Ala or Gly;

Al8 is Ser or Thr;

A24 is Gln or His;

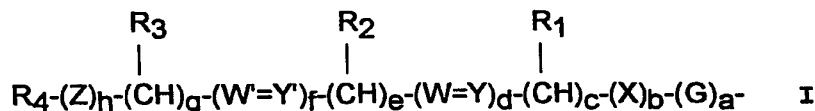
A27 is Met, Ile or Nle;

A28 is Ser or Asp;

A30 is any amino acid sequence of 1 to 15 residues or absent;

R₀ is NH₂ or NH-(CH₂)_n-CONH₂, with n=1 to 12;
and

wherein Al is N- or O-anchored by a hydrophobic tail of the following general formula I:



wherein,

G is a carbonyl, a phosphonyl, a sulfonyl or a sulfinyl group;

X is a oxygen atom, sulfur atom or an amino group (NH);

(W=Y) represents cis or trans (CH=CR₅);

(W'=Y') represents cis or trans (CH=CR₆);

Z is an oxygen or a sulfur atom;

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R₁, R₂ and R₃, independently, are selected from a hydroxyl group, a hydrogen atom, and a linear or branched C₁-C₆ alkyl group;

R₄ is an hydroxyl group, a hydrogen atom or a linear or branched C₅-C₉ alkyl group;

R₅ and R₆, independently, are a hydrogen atom or a linear or branched C₁-C₄ alkyl group;

a is 0 or 1;

b is 0 or 1;

c is 0 to 8;

the sum of d + f is ≥ 1 ;

e is 0 to 8;

g is 0 to 8;

h is 0 to 1;

wherein the sum of a, b, c, d, e, f, g and h is such that the hydrophobic tail of formula I has a linear main chain of between 5 and 8 atoms (C, O and/or S).

2. The chimeric fatty body-pro-GRF analog of claim 1, wherein A₁ is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein both a and b = 1; the sum of d + f is ≥ 1 ; h = 0; G = carbonyl; X = oxygen atom; R₁, R₂, R₃, R₄ = hydrogen atom and the sum c + e + g = 3, 4, 5 or 6.

3. The chimeric fatty body-pro-GRF analog of claim 1, wherein A₁ is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein a = 1; the sum of d + f is ≥ 1 ; G = carbonyl; R₁, R₂, R₃ and R₄ = hydroxyl group and the sum c + e + g = 4, 5, 6 or 7.

4. The chimeric fatty body-pro-GRF analog of claim 1, wherein the sum d + f = 1.

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5. The chimeric fatty body-pro-GRF analog of claim 4, wherein A1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein $a = 1$; each of b and $h = 0$; G = carbonyl; R_1 , R_2 , R_3 and R_4 = hydrogen atom and the sum $c + e + g = 2, 3, 4$ or 5 .

6. The chimeric fatty body-pro-GRF analog of claim 5, wherein c is 0.

7. The chimeric fatty body-pro-GRF analog of claim 6, wherein A30 is absent or Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu.

8. The chimeric fatty body-pro-GRF analog of claim 7, wherein A30 is absent and R_0 is NH_2 .

9. The chimeric fatty body-pro-GRF analog of claim 8 of the formula $cisCH_3-(CH_2)_2-CH=CH-CO-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-NH_2$ or $transCH_3-(CH_2)_2-CH=CH-CO-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-NH_2$.

10. The chimeric fatty body-pro-GRF analog of claim 7, wherein R_0 is NH_2 .

11. The chimeric fatty body-pro-GRF analog of claim 10, of the formula $cisCH_3-CH_2-CH=CH-CH_2-CO-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH_2$ or $transCH_3-CH_2-CH=CH-CH_2-CO-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-$

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Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH₂.

12. The chimeric fatty body-pro-GRF analog of claim 1, wherein A1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein $a = 1$; each of b and h = 0; the sum $d + f = 2$; G = carbonyl; R₁, R₂, R₃ and R₄ = hydrogen atom and the sum $c + e + g = 0, 1, 2$ or 3.

13. The chimeric fatty body-pro-GRF analog of claim 1, wherein A1 is Tyr or His N-alpha anchored by hydrophobic tail of formula I, wherein $a = 1$; the sum of d + f is ≥ 1 ; h = 0; G = carbonyl; R₁, R₂, R₃ and R₄ = hydrogen atom; and the sum $c + e + g = 4, 5, 6$ or 7.

14. A pharmaceutical formulation for inducing growth hormone release which comprises as an active ingredient a GRF analog as claimed in claim 1, in association with a pharmaceutically acceptable carrier, excipient or diluent.

15. A method of increasing the level of growth hormone in a patient which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1.

16. A method for the diagnosis of growth hormone deficiencies in patients, which comprises administering to said patient a GRF analog as claimed in claim 1 and measuring the growth hormone response.

17. A method for the treatment of pituitary dwarfism or growth retardation in a patient, which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1.

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18. A method for the treatment of wound or bone healing in a patient, which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1.

19. A method for the treatment of osteoporosis in a patient, which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1.

20. A method for improving protein anabolism in human or animal, which comprises administering to said human or animal an effective amount of a GRF analog as claimed in claim 1.

21. The use of a GRF analog as claimed in claim 1 for increasing the level of growth hormone in a patient.

22. The use of a GRF analog as claimed in claim 1 for the treatment of pituitary dwarfism or growth retardation in a patient.

23. The use of a GRF analog as claimed in claim 1 for the treatment of wound or bone healing in a patient.

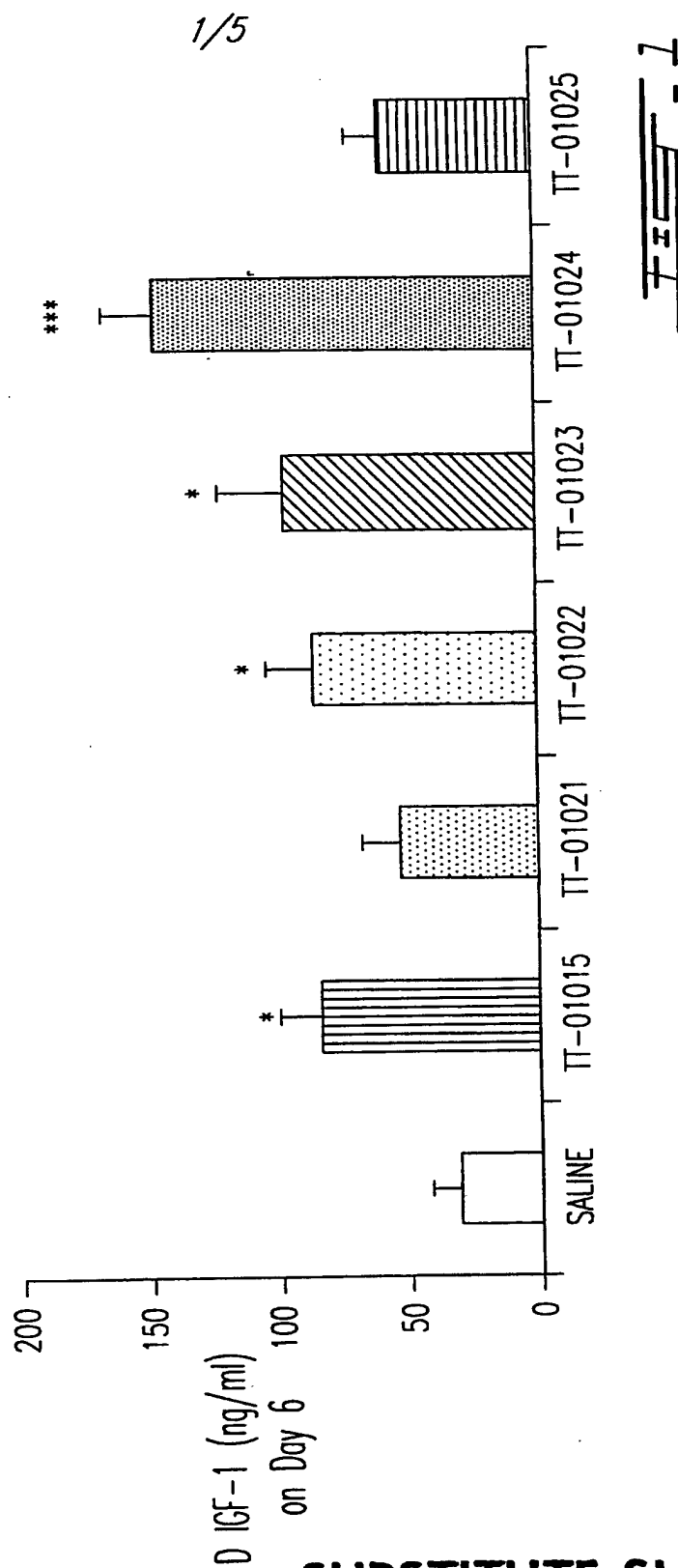
24. The use of a GRF analog as claimed in claim 1 for the treatment of osteoporosis in a patient.

25. The use of a GRF analog as claimed in claim 1 for improving protein anabolism in human or animal.

AMENDED SHEET (ARTICLE 19)

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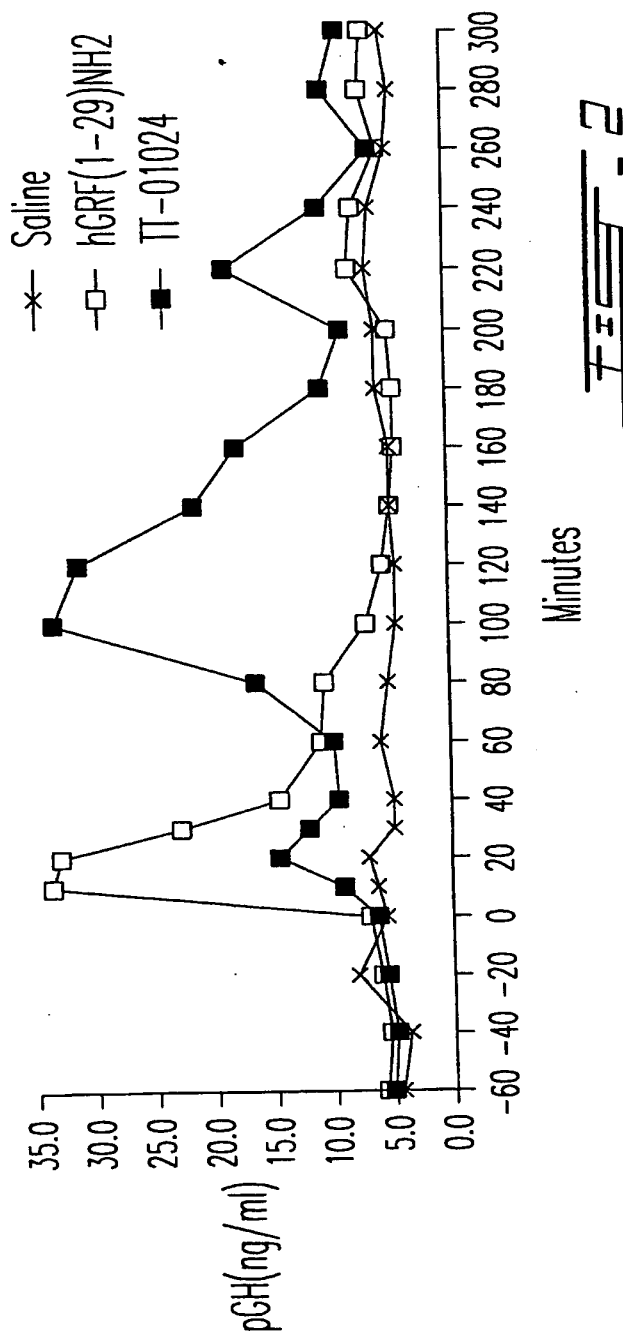


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Minutes

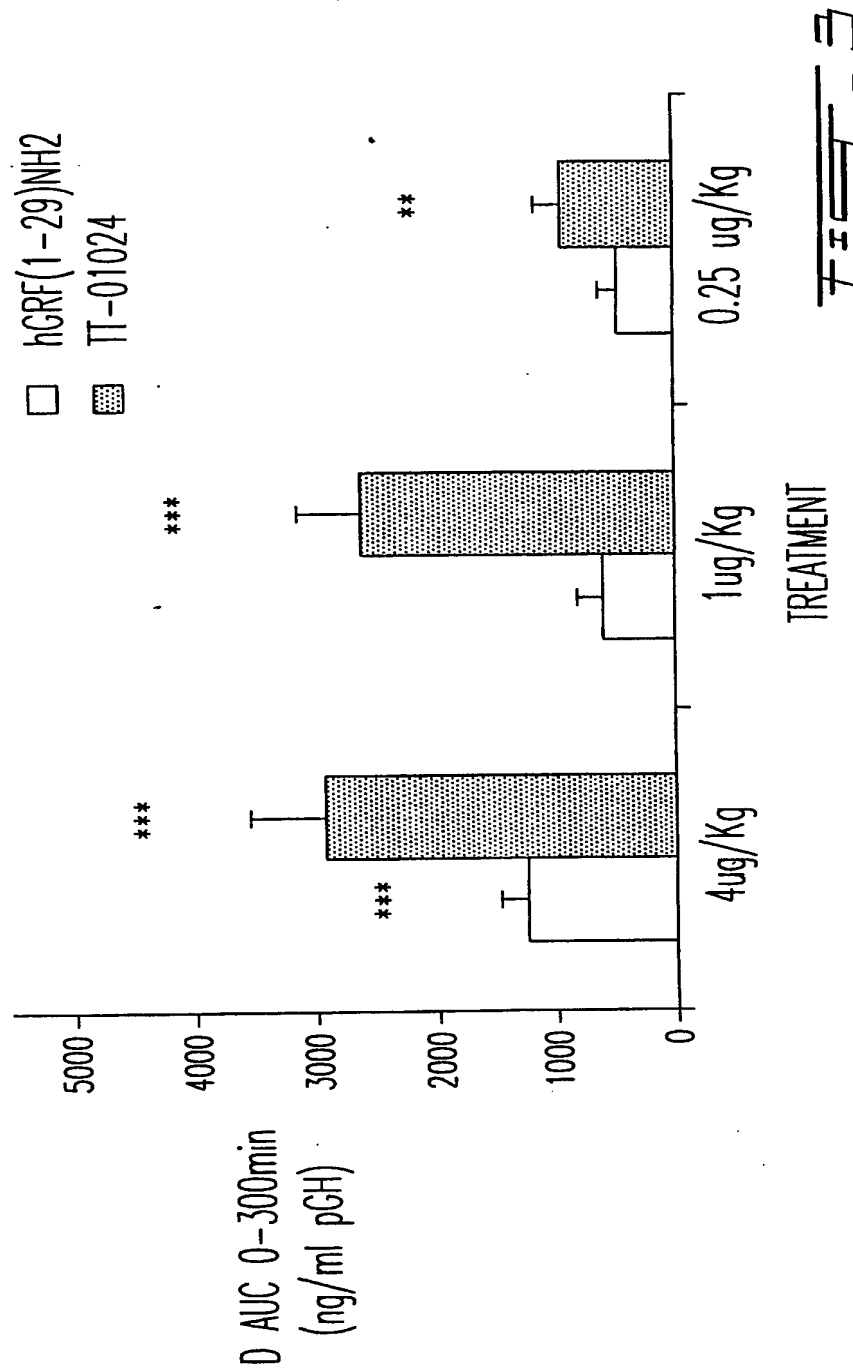
Figure 2

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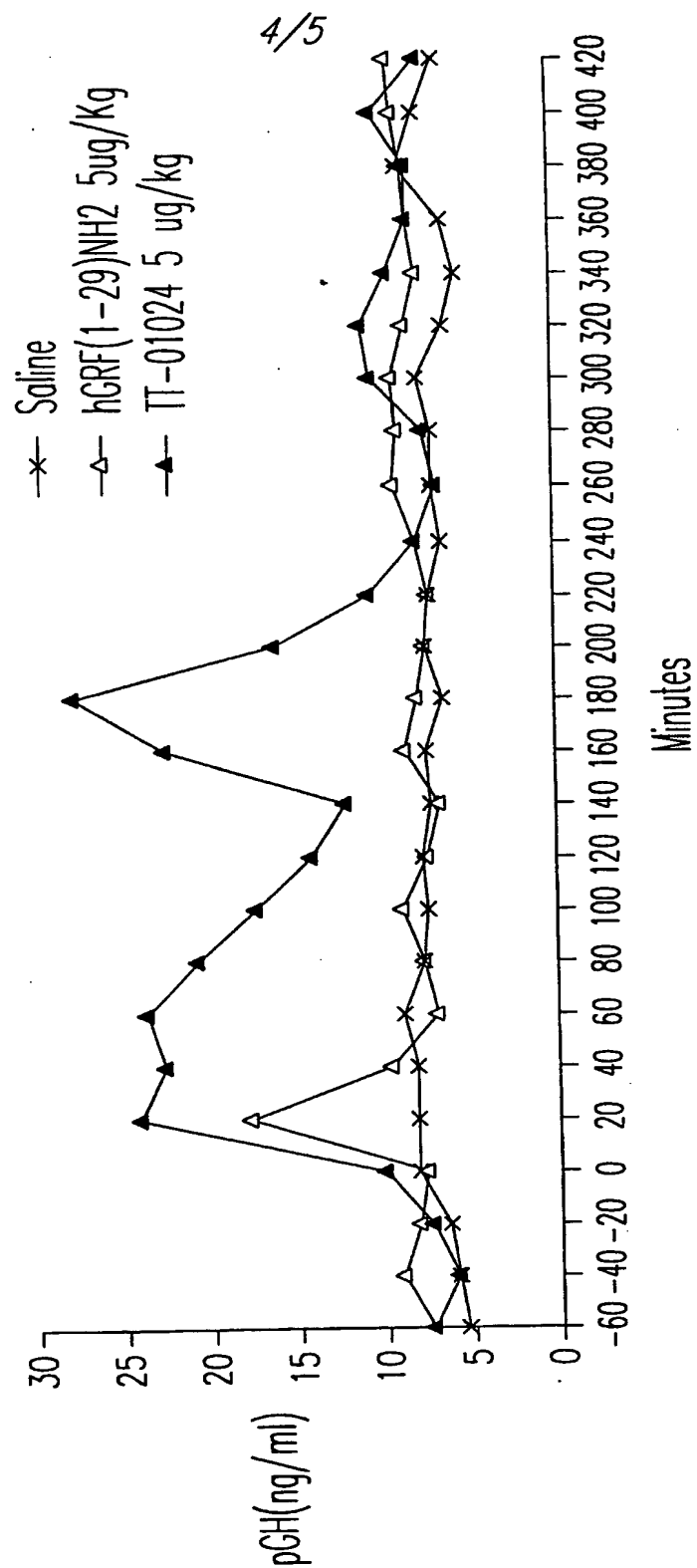
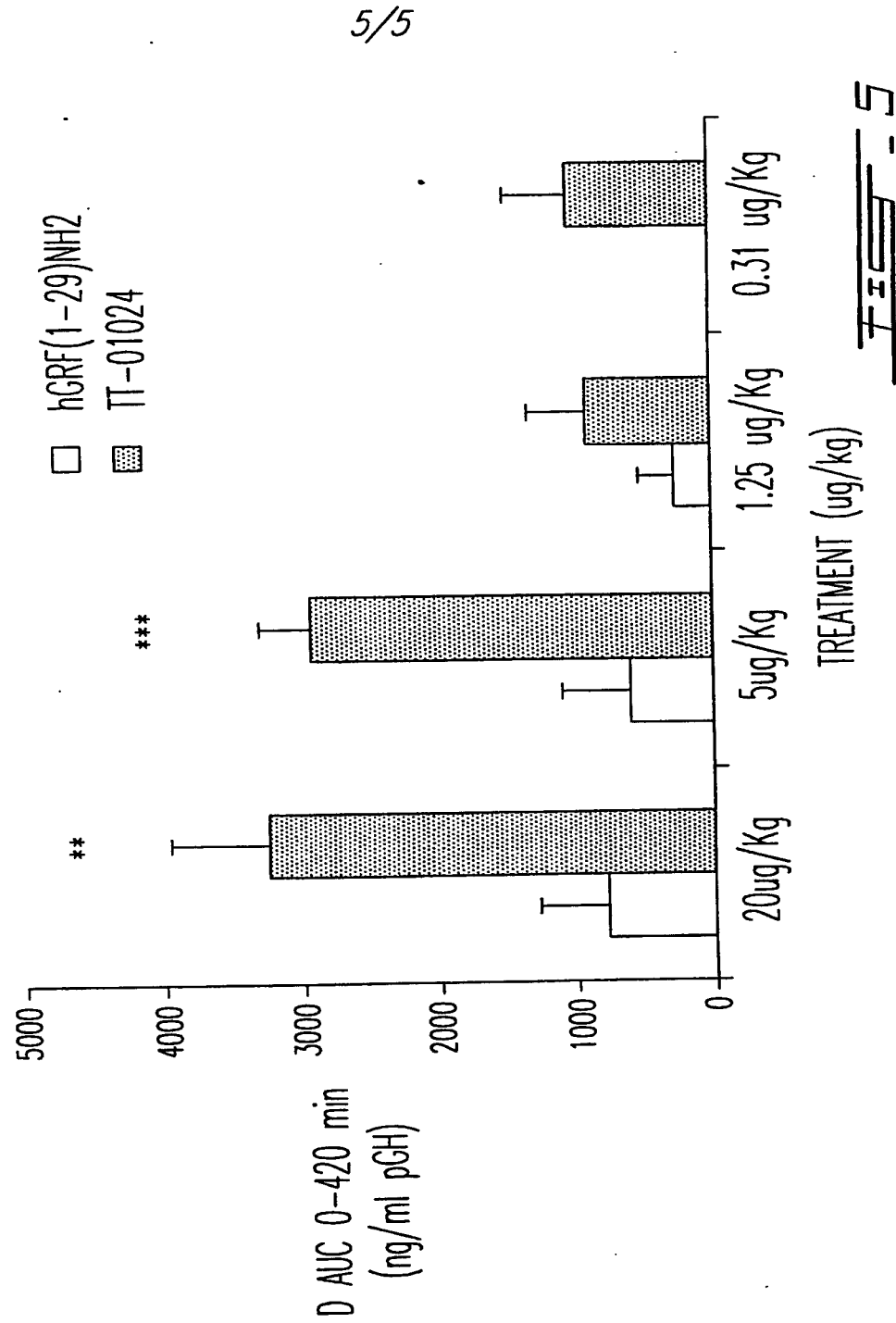


FIG - 4

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SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No

PCT, CA 96/00327

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C07K14/60 A61K38/25 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 314 866 (UNIV TULANE) 10 May 1989 see claims; examples ---	1-13
X	EP,A,0 320 785 (HOECHST AG) 21 June 1989 see claims; examples ---	1-13
X	EP,A,0 511 003 (LILLY CO ELI) 28 October 1992 see claims; examples ---	1
A	EP,A,0 188 214 (UNIV TULANE) 23 July 1986 see claims; examples -----	1-13



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

30 August 1996

Date of mailing of the international search report

06.09.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+ 31-70) 340-3016

Authorized officer

Fuhr, C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA96/00327

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORTInternational Application No. **PCT/CA96/00327****FURTHER INFORMATION CONTINUED FROM PCT/SA/210**

Remark: As far as claims 7 - 12 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound and/or composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 96/00327

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0314866	10-05-89	AU-B- 613364	01-08-91
		AU-B- 1589888	17-11-88
		IL-A- 86102	12-04-94
		JP-A- 63287799	24-11-88
		US-A- 5112808	12-05-92
		ZA-A- 8803307	14-11-88
EP-A-0320785	21-06-89	DE-A- 3742633	29-06-89
		AU-B- 2684388	22-06-89
		JP-A- 1199997	11-08-89
EP-A-0511003	28-10-92	AU-B- 1513292	29-10-92
		AU-B- 2333495	14-09-95
		CA-A- 2067304	27-10-92
		CN-A- 1068123	20-01-93
		HU-A- 64589	28-01-94
		JP-A- 5279388	26-10-93
		NZ-A- 242459	23-12-93
EP-A-0188214	23-07-86	AU-B- 590738	16-11-89
		AU-B- 5229986	24-07-86
		CA-A- 1271600	10-07-90
		JP-A- 61210099	18-09-86
		US-A- 5416073	16-05-95